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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1623219> since 2018-05-16T13:42:38Z

Published version:

DOI:10.1155/2012/564121

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Review Article

Human Biomonitoring of Engineered Nanoparticles: An Appraisal of Critical Issues and Potential Biomarkers

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Received 5 January 2012; Revised 26 April 2012; Accepted 26 April 2012

Academic Editor: Ivo Iavicoli

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The present paper deals with the applicability of biological monitoring to the assessment of exposure and possible effects deriving from exposure to engineered nanomaterials (NM). After establishing a conceptual framework in which human biomonitoring should be placed, the paper reviews the critical issues related to the unusual properties of NM affecting the implementation of biomonitoring activities for this new class of chemicals. Relying on the recent advances in the toxicogenomic, it is possible to assess whether specific biological pathways are activated or perturbed by specific NM. However, to evaluate if quantitative changes in these biomarkers can be used as indicators or predictors for toxicity in humans, validation on well characterised groups of exposed people is needed. At present, it appears more pragmatic to evolve NM-associated biomarker identification considering relevant biological responses found in environmental and occupational studies and assessing the early events associated with exposure to these NM. The battery of biochemical markers includes soluble molecules, antioxidant capacity, peroxidated lipids and carbonyl groups in serum proteins as a biomarkers of systemic inflammation and vascular adhesion molecules to assess endothelial activation/damage. Abnormalities in exhaled breath condensate chemistry reflecting intrinsic changes in the airway lining fluid and lung inflammation seem promising tools suitable for BM studies and are broadly discussed.

1. Definition and Meaning of Human Biological Monitoring

Biological monitoring (BM) has been defined in different ways, according to the purpose and the context [1–6]. In occupational health, BM deals with the systematic or repetitive measurement of chemical or biochemical markers in fluids, tissues, or other accessible samples from people exposed to or with past exposure to chemicals risk factors. The main objectives of such periodical measurements are (a) the assessment of individual or group exposure; (b) ensuring health protection by identifying early, specific nonadverse biological effect parameters which are indicative, if compared with adequate reference values, of an actual or potential condition leading to health damage; ultimately (c) the assessment of health risk to exposed subjects [2, 3]. BM can be used as a valid tool in the practice of occupational safety and health with the purpose of identifying potential hazards of new and emerging chemicals, including manufactured nanoparticles, and thus identifying groups at higher risk of health outcomes [2].

BM is becoming more popular as the number of biomarkers increases and the number of successful examples of field application proves the validity of such biomarkers. Biomarkers are regarded as early, preferably reversible, biological signs which are indicative, if compared with adequate reference values, of an actual or potential condition of exposure, effect, or susceptibility possibly resulting in health damage or disease [3]. Biomarkers are increasingly used as surrogate indicators of designated events in a biological system due to the inaccessibility of target organs; in spite of this limitation, it is thought that biomarkers are more directly related to the adverse effects which one attempts to prevent than any ambient measurement [4]. Depending on their toxicological significance, biomarkers have been classified in three main categories: biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility. A biomarker of exposure has been defined as “an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism” [5]. Providing objective demonstration of the absorption of

chemicals in the body, exposure biomarkers can be useful in occupational toxicology for a more accurate risk assessment, reducing misclassification in epidemiological studies, and modelling internal dose.

A biomarker of effect is “any measurable biochemical, physiological or other alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease” [5]. Biomarkers of effect can be used in health surveillance programs aimed at the early diagnosis of exposure-related (or associated) diseases, but the application of effect monitoring is most often used to evaluate whether a well-characterized exposure is associated with a shift in the distribution of relevant biochemical or functional endpoints indicative of early changes in the target or critical organs/tissue. Biomarkers of susceptibility, including both inherent (e.g., genetic polymorphisms of toxicologically relevant phenotypes of drug metabolizing enzymes or DNA repair enzymes) and acquired conditions [5], should help to distinguish groups at risk at the same exposure levels, but they should be used as effect modifiers rather than as risk factors.

For preventive purpose, biomarkers should not be considered as diagnostic tests but rather as indicators reflecting early modifications preceding progressive structural or functional damage at the molecular, cellular, and tissue level, that is, changes possibly leading to adverse effects but completely reversible upon the removal from the exposure of concern. In order to be useful in risk assessment, a biomarker should provide relevant information on important issues concerning health risks and valid from both analytical and epidemiological aspects [6]. Validity relies on the intrinsic characteristics of the biomarker, due to its pathophysiological meaning, that is, the ability to reflect, even subtle, changes in a biological system; in particular, external validity is required to obtain results which can be generalized to other populations [2].

2. Critical Issues of Biomonitoring for Engineered Nanomaterials

The development of appropriate biomarkers requires the knowledge of pharmacokinetic and pharmacodynamic data for a given chemical, which are still lacking for many classes of NMs, in spite of the great development of *in silico* models [7]. The internal dose of a given chemical is usually assessed by both the amount of the chemical and/or its metabolites and the products of interaction with target biomolecules (DNA, proteins). For some metal nanoparticles, fullerenes and Single-Wall Carbon Nanotubes (SWCNTs), the available biokinetic data [8, 9] reveal appreciable translocation rates from the lung interstitium to bloodstream and secondary organ. Although such mechanisms can potentially explain the manifestations attributable to UFP exposure, at present, it is unknown whether it could represent a causal pathway for NM entailing nano-specific outcomes.

As compared to other chemical substances, NMs do not undergo biotransformation leading to moieties, or break, down by, products; on the other hand, the particle

opsonisation by serum or membrane proteins, the specific proteins coating the nanoparticle surface (“corona” effect), is one of the key factors determining the fate and outcomes of nanomaterial interacting with biological systems [10, 11].

A specific type of interaction occurs when proteins adsorb, and this interaction affects both the particle and the protein function. For instance, 14 nm carbon black (CB) significantly adsorbs the cytokines IL-8 and TNF-alpha compared with a similar dose of 260 nm CB [12]. TNF alpha bound to 14 nm CB induced a level of ICAM-1 expression that was no greater than the control level, suggesting that the TNF alpha activity may be inhibited.

Metal nanoparticles like cobalt [13], iron oxide [14] and zinc oxide, silver [15] can release metal ions, and the role of particle dissolution in biological media, as well as its prevention by doping particle surface, has paid attention for the clearance of the particle out of the deposition sites and for the effects of particles in target organs [8, 14, 16]. Agglomeration and solubility largely affect the biological behaviour of NMs and, ultimately, their organ distribution; low-soluble CNT or TiO₂ nanoparticles show biopersistence, and their slow clearance lead to the tendency to accumulate over time with exposure [8, 9]. Although some NMs have demonstrated to easily cross biological barriers, the magnitude of NMs into the systemic circulation could be negligible to lead significant internal dose. Analytical capabilities now permit quantization of “background levels” in people not knowingly exposed, but the toxicological significance of these low doses needs to be carefully assessed [17].

Data generated by toxicological tests can be used to establish a quantitative relationship between the different physico-chemical characteristics of nanoparticles and the response elicited [18]. For nanoparticles, controversies exist about the best descriptor of dose related with biological effects, particle number, and surface area being the best dose metrics for NMs both *in vitro* and *in vivo*. [19–21]. The unpredictable behaviour of several NMs would require to assess the dose-response relationship of the most sensitive body organ rather than solely on the organ through which nanoparticles enter the body. Although understanding target organ dose is still problematic, new methods for tracking and measuring NMs *in vivo*, currently applied to assess the effectiveness of dose delivered at target cells [22, 23], could provide effective tools for obtaining quantitative data that are essential for dosimetry model validation and, ultimately, for ascertaining the range of exposure level with no observed adverse effects (NOAELs).

3. Identifying Relevant Effects and Mechanisms

To develop biomarkers suitable for human studies involving people exposed to NMs, it has been suggested to use a step-wise approach to evolve nanoparticle-associated biomarker identification from biochemical, cellular, and animal studies, the same approach that has been used for studying air pollution-associated biomarkers [24]. Inhaled ultrafine particles (UFPs) and combustion-derived nanoparticles are

capable of inducing oxidative stress in the lung as well as in systemic circulation and are involved in many adverse effects or pathological conditions associated with respiratory and cardiovascular disease outcomes. For environmental particles, oxidative stress and changes to biomolecules may arise from direct generation of reactive oxygen species from the surface, soluble compounds, such as, transition metals or organics, altered function of mitochondria or NADPH-oxidase, and activation of inflammatory cells capable of generating ROS and reactive nitrogen species [25].

It should be emphasized that incidental UFPs, mostly generated by combustion sources, are heterogeneous in size and composition (including organics undergoing metabolic activation and producing reactive species, such as, polynuclear aromatic hydrocarbons, elemental carbon and metals), whereas NMs are intentionally made of defined composition and surface chemistry. Although similar in size, ambient and engineered nanoparticles may significantly differ for biological activity and toxicological properties. Since ambient UFPs are one of the components of a complex mixture of pollutants including oxidant gases and biocontaminants, it is difficult to attribute specific health outcomes to a specific class of contaminants.

Recent research has identified the most significant biological responses and target organ affected by different NM [18, 26–28] and the toxicity paradigms have been elucidated [15, 29, 30].

Figure 1 summarizes some potential important factors in correlating exposure, dosimetry, and health effects of inhaled nanoparticles. Since nanoparticles have a greater potential to deposit and target both the upper and lower regions of the respiratory system and are capable to induce more oxidative stress and inflammation than their fine-sized counterparts, possibly leading to long-term effects, this justifies the study on their health impact [28].

Epidemiological studies have found hazardous respiratory effects from occupational exposure to some industrial processes involving generation of significant amounts of UFP, such as, carbon black [31], fumed silica [32], and metal oxide [33, 34]. However, also some few NMs, such as, titanium dioxide (TiO_2), copper oxide (CuO), ZnO and iron oxide nanoparticles, cationic polystyrene, and C60 fullerene have demonstrated to have prooxidative and proinflammatory properties both *in vitro* and *in vivo*, mainly related to their surface reactivity and chemical composition [28], although not all NPs cause inflammation via a mechanism involving oxidative stress. Experimental studies have characterized tissue response to carbon nanotubes (CNTs) and showed that deposition following instillation or inhalation leads to transient inflammatory changes, lung tissue remodelling, and fibrosis [35, 36].

Since NMs can potentially affect multiple cellular functions and interfere with many components of the cell machinery [37], it is difficult to determine with conventional assay what are changes and adverse effects that may occur, and whether biochemical and/or functional changes can be material-specific or exposure-route specific. Conventional toxicology has used surrogate markers that are correlated

with toxic responses to monitor adverse outcomes in inaccessible tissues. An alternative approach is suggested by system toxicology, which postulates that there are size-specific, shape-specific, and surface-specific effects and effectors for particles at the quantum range, which are different from the effects observed for micro-sized particulates [37–39].

The process of determining the relationship between a particular expression profile and the toxicological phenotype of the organism for a particular exposure or dose and at a particular time is called “phenotypic anchoring”. This approach can be used in conjunction with lower doses of the toxicant to classify agents and to explore the mechanisms of toxicity, for example, by assessing transcriptional changes occurring after exposure to both low- and high-dose of NMs, and detecting the biological responses by using transcriptome measurements. Toxicogenomics approach has been used in assessing specific mechanisms at the molecular level for silicon dioxide, dendrimers, and carbon nanoparticles, for example, SWCNT, Multi-Wall carbon Nanotubes (MWCNTs), carbon nanooxions and fullerenes, and metal nanoparticles (e.g., copper and silver), identifying patterns of cellular perturbations in specific pathways, through identification and quantification of global shifts in gene expression in cell models challenged with NMs [38–42].

SWCNT may induce cell apoptosis, which is characterized by upregulation expression of apoptosis-associated genes (e.g., *p16*, *bax*, *hrk*, *bak1*, *p57*, *FGFR2*, *TGF beta receptor 1* and *TNFAIP2* genes) and downregulation expression of cell-cycle-associated genes (such as *cyclin D1*, *cdk2*, *cdk4*, and *cdk6*) as compared to normal cells [40]. Silica nanoparticles induced a higher level of acute phase proteins, such as, haptoglobin, C-reactive protein (CRP), and serum amyloid A (SAA) than larger silica particles (diameter >100 nm) after intravenously treatment; acute phase proteins, such as haptoglobin were significantly elevated in plasma of mice exposed to silica nanoparticles with a diameter of 70 nm (nSP70) compared to normal mice and those exposed to silica particles with a diameter of 1000 nm. Furthermore, the CRP and SAA were also elevated in plasma of nSP70-treated mice. In addition, the level of these acute phase proteins was elevated in the plasma of mice after intranasal treatment with nSP30 [42].

Whole genome microarray analysis of the early gene expression changes induced by 10 and 500 nm amorphous silica nanoparticles showed that the magnitude of change for the majority of genes affected correlated more tightly with particle surface area than either particle mass or number, and gene expression changes were particle size specific [41]. However, the overall biological processes represented by all gene expression changes were nearly identical, irrespective of particle diameter, suggesting that on an equivalent nominal surface area basis, common biological modes of action are expected for nano- and supranano-sized silica particles. The most robust transcriptional change observed was an induction of the *Cxcl2* chemokine (*MIP-2a*), also implicated in crystalline silica-induced lung neutrophil recruitment [41].

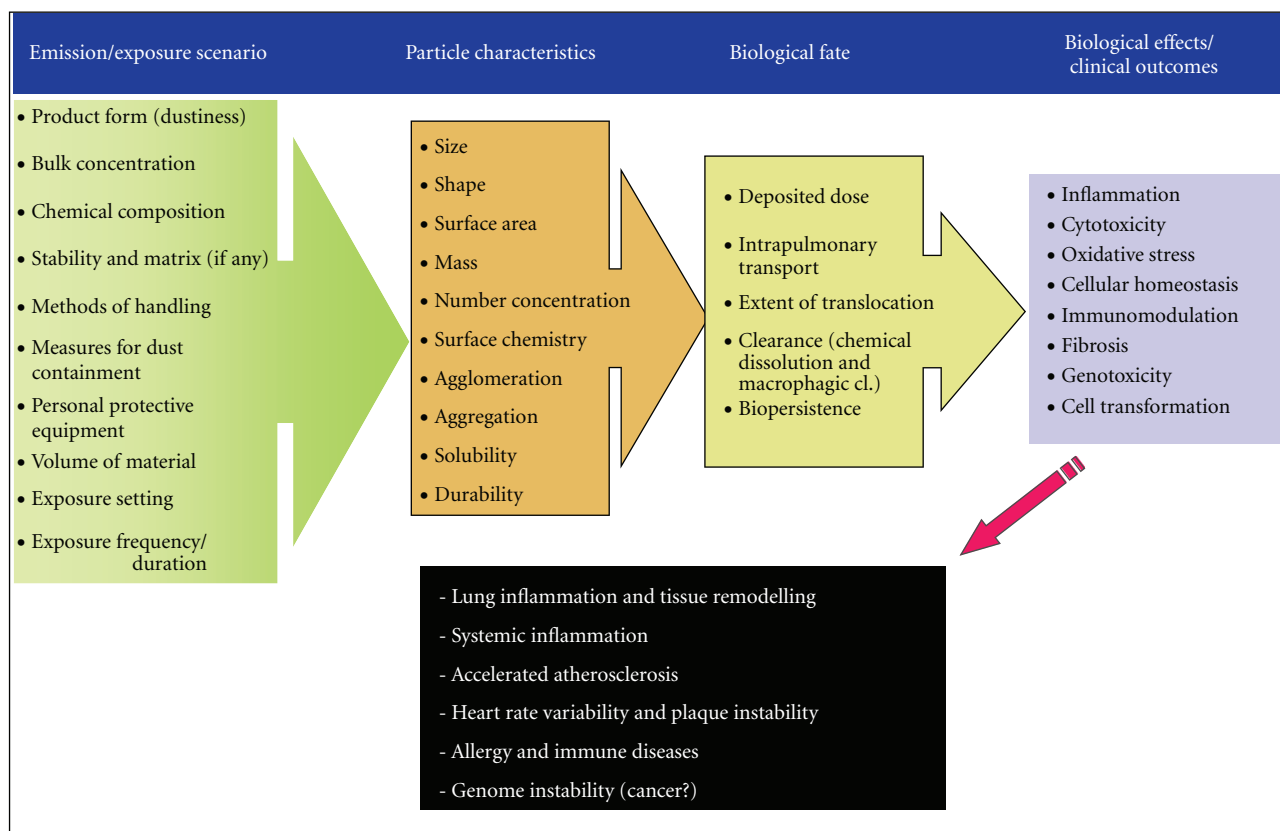


FIGURE 1: Potential important factors in correlating exposure, internal dose, and health effects of nanoparticles.

Pulmonary exposure to CNT resulted in an elevated series of measurable potential biomarkers in blood, including, genes expressed in the circulating blood cells and/or soluble proteins not unique to the type of particles [43]. In particular, MWCNT induced gene upregulation of more than half of the tested genes in the lung related to inflammation, oxidative stress, coagulation, and tissue remodelling and in a significant increase in the circulating blood gene expression of several biomarkers of neutrophil response. Interestingly, several genes were activated in the circulating blood cells but not in the lung at least at the 4h after pulmonary exposure to MWCNT, for example, osteopontin, colony stimulating factor-1 (*CSF-1*), and insulin growth factor receptor 1 (*IGF-1R*). Pulmonary exposure to CNT also triggered the induction of primary cytokines, such as IL-6 and IL-1 β , which regulate multiple pathways of the inflammatory cascade as well as secondary inflammatory mediators and chemokines which directly regulate leukocyte recruitment to the inflammatory site (32). Teeguarden et al. [44] identified a pattern of 109 proteins representing cellular processes affected by both SWCNT and crocidolite asbestos; in particular, one high-sensitivity marker of inflammation (*S100a9*) may represent a promising biomarker of human response to SWCNT exposure.

High-throughput toxicological studies thus support the existence of both nanoscale effects and specific molecular effectors differentiating nanotoxicology from conventional

toxicology. Figure 2 summarizes such system-toxicology-based integrative approach to the identification of biomarkers. The perspective of identifying fingerprints of selected NMs by “-omic” techniques (i.e., metabolomic analysis, toxicogenomic, oxidative lipidomics based on mass spectrometry-based profiling of lipid profiles of exposed cells and tissues) applied to nanotoxicology will allow to assess whether particular biological pathways are activated or perturbed by specific nanoparticles and to evaluate if the quantitative changes in these biomarkers can be used as indicators or predictors for toxicity in humans.

4. Biomarkers of Lung and Systemic Inflammation

To assess the early events preceding progressive structural or functional damage at the molecular, cellular, and tissue level associated with exposure to NMs, the choice of potential biomarkers to be studied can include the changes that indicate local and systemic oxidative stress, systemic inflammation, and inflammatory response in target organs, such as, those in respiratory and cardiovascular systems. Although inflammatory pathways are the main mechanism investigated in relation to cardiovascular outcomes, other mechanisms triggered by UFPs exposure, such as, the involvement of vagal bronchopulmonary receptors and neuronal pathways initiated in the lung, should be considered [45].

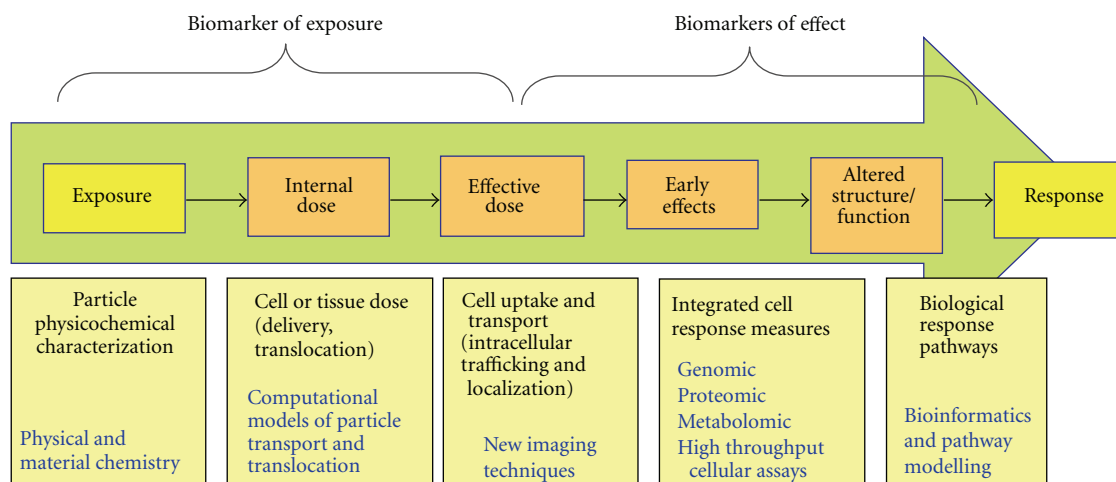


FIGURE 2: System toxicology-based integrative approach to the identification of biomarkers. Recent advances in “-omics” and new imaging techniques describing the cellular fate of NMs may lead to identify fingerprints of selected NMs, to assess whether particular biological pathways are activated or perturbed by NMs, and to evaluate if the quantitative changes in these biomarkers can be used as indicators or predictors for toxicity in humans.

Quantification of lung inflammation is currently based on invasive methods, including, the analysis of bronchoalveolar lavage fluid (BALF), bronchoscopy and bronchial biopsies, semi-invasive methods, such as, sputum induction, and the measurement of inflammatory biomarkers in plasma and urine which are likely to reflect systemic rather than lung inflammation [46]. Breath analysis has been suggested as a promising noninvasive approach that allows the identification of the inflammatory and oxidative stress biomarkers involved in the pathogenesis of various respiratory conditions both for clinical applications [46] and for investigating occupational lung diseases [47, 48].

Different biomarkers of effect, reflecting oxidative stress and inflammatory pathways, can be determined in EBC [46]; for instance, Thiobarbituric Acid Reactive Substances (TBARSs), such as, malondialdehyde, produced by lipoperoxidation of membranes, and 8-isoprostane, a peroxidation products of prostaglandin metabolism, can be quantified; changes in pH, proteins, and proinflammatory cytokines, such as, leukotrienes B₄ (LTB₄) can also be determined as biomarkers of inflammation. Inflammatory response in the airways is characterized by an influx of neutrophils, whose activation is associated with a respiratory burst resulting in overproduction of hydrogen peroxide (H₂O₂), which is measurable in EBC. Glutathione (GSH), which is present in high concentration in epithelial lining fluid and at tissue level of the lung, represents one of the major biological antioxidant defence mechanisms against reactive oxygen species and the secondary products ROS reacting with thiol groups can severely deplete the GSH pool.

Various classes of volatile organic compounds (VOCs) can also be measured in exhaled breath, including, the saturated hydrocarbons and oxygen-containing substances formed during the fatty acid lipid peroxidation of cell membranes [49].

A wide variety of carbonyl compounds are generated as secondary oxidation products during respiratory burst. In

particular, saturated aldehydes, such as hexanal, heptanal, and nonanal are formed by the peroxidation of omega-3 and -6 fatty acids (PUFAs), the basic components of cell membrane phospholipids. Volatile aldehydes are poorly soluble in blood and are therefore excreted into the breath within minutes of their formation in tissues, making it possible for the study of pulmonary diseases associated with inflammatory processes (e.g., COPD, asthma, interstitial lung disease) and the early diagnosis of lung cancer [50, 51].

Nitric Oxide (NO) is produced by all cellular components of pulmonary inflammation (macrophages, epithelial cells, mast cells, lymphocytes, and granulocytes). Studying NO exhaled from the lower airways offers a unique possibility to study features of pulmonary NO metabolism noninvasively in lung inflammatory states [52]. Under physiologic conditions, NO is unstable, reacting with oxygen to form oxides of nitrogen (NO_x), such as, nitrite (NO₂⁻) and nitrate (NO₃⁻) and with superoxide anion to form the potent oxidant peroxynitrite (ONOO⁻), which reacts with a wide variety of compounds, including, DNA, cellular lipids, and sulphhydryl groups on proteins thus promoting nitrosative stress. Nitrosothiols (RS-NOs), which are formed by interaction of nitric oxide (NO) with GSH, can be reliably used as biomarkers in different inflammatory airway diseases [53]. Nitrite and nitrate in EBC have been used in evaluating the role of oxidative stress in acute responses to exposures that occur inside vehicles or during near-roadway activities [54].

A broad panel of circulating biomarkers reflecting inflammation endpoint, platelet activation, and antioxidant capacity by the copper-/zinc-superoxide dismutase (Cu, Zn-SOD) and glutathione peroxidase-1 (GPx-1) activity has been investigated in studies on air pollution and health effects. Delfino et al. have assessed the relationship between exposure to particles of 0.25 μm in aerodynamic diameter (PM_{0.25}), organic carbon, and elemental carbon from outdoor sources and changes in circulating biomarkers

of systemic inflammation [55, 56]. These studies showed the inactivation of antioxidant enzymes within erythrocytes by traffic-related pollutant components, including, polycyclic aromatic hydrocarbons, among a subgroup of people. Plasma interleukin-6 (IL-6) and soluble tumor necrosis factor-receptor II (sTNF-RII) were investigated during a longitudinal study considering both indoor and outdoor exposure and showed a positive association with vehicle emissions tracer. High-sensitivity C-reactive protein (hsCRP) in plasma, plasma fibrinogen, interleukin-6 (IL-6) could represent the best candidate for human BM following NM exposure.

Radomski et al. [57] showed that both urban dusts and engineered carbon particles, such as, CNT and carbon black, except C₆₀CS, stimulated platelet aggregation and accelerated the rate of vascular thrombosis in rat carotid arteries with a similar rank order of efficacy. All particles resulted in upregulation of GPIIb/IIIa in platelets. Similarly, exposure to nano- Ag (0.05–0.1 mg/kg i.v. or 5–10 mg/kg intratracheal instillation) *in vivo* enhanced platelet aggregation and promoted venous thrombus formation in rats [58]. Single-walled carbon nanotubes (0.01–1 mg/kg) were injected into anesthetized mice, induced platelet activation *in vitro*, and exerted prothrombotic effects in the microcirculation *in vivo*, whereas similar dose of titanium dioxide (1 mg/kg) did not result in thrombus formation [59].

In order to assess early systemic prothrombotic effects induced by fine particle (<2.5 μ m) exposure, quantification of tissue factor (TF) activity in plasma, which is expressed in subendothelial cells upon injury or on the surface of circulating monocytes, macrophages and neutrophils, and the levels of plasminogen activator inhibitor-1 (PAI-1), can also be proposed as biomarkers [60].

The endothelium plays an important role in maintaining the vascular homeostasis by producing vasoactive factors that regulate the tone of the vascular system in response to cell surface receptor stimulation or mechanical stress but also in the regulation of vascular inflammation and thrombosis. NMs can directly affect endothelial cells metabolism via oxidative stress generation and endothelial repair capacity [61, 62]. For instance, exposure to ultrafine TiO₂ particles can affect vasomotor function with minimal pulmonary effects [63]. Vesterdal et al. [64] showed that exposure of young and aged apolipoprotein E knockout mice (apoE^{-/-}) to CB (Printex 90, 14 nm average size) by intratracheal instillation, resulted in modest vasomotor impairment, with a lack in association with nitrosative stress (3-nitrotyrosine) and without increases in the expression of vascular adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) on endothelial cells or in plaque progression.

5. Biomarkers of Oxidative Stress and DNA Damage

Panel studies and cross-sectional investigations on health effects of particulate matter exposure have found consistent associations between exposure to combustion-derived particles and products of oxidative damage of DNA and lipids

[65–67]. Among the DNA oxidation products, such as 8-oxo-7,8-dihydroguanine (8-oxoGua) or the corresponding deoxynucleoside 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) measured in DNA and urine, the exocyclic M1 adduct to guanine (M1dG) was the most studied. Among the biomarkers of lipid peroxidation products measured in EBC, blood, or urine, both conjugated dienes (CDs), lipid hydroperoxides, malondialdehyde (MDA), Thiobarbituric Acid Reactive Substances (TBARSs), and F2-isoprostanes have been assessed. Base excision repair of oxidative damage to DNA measured in urine, such as, 8-hydroxy-2'-deoxyguanosine (8OHdG), seems to originate mostly from the oxidation of the deoxynucleotide pool and does not represent solely repairing/excretion of the oxidized DNA guanine. Once produced, 8OHdG is very stable and is not further metabolized in the systemic circulation. After exposure to oxidants, the repair and final 8OHdG excretion in urine is rapid, that is, within at least 24 hours [68]. Increased 8OHdG urinary levels within each shift and between days have been shown in workers exposed to low levels of respirable PM₄ (range 25–71 μ g/m³) [69].

Whereas the DNA damaging potential of many nanoparticles is known, *in vitro* studies have demonstrated that NMs of different composition (metal/metal-oxide nanoparticles, silica, quantum dots, fullerenes, nanofibers) can alter DNA structure by oxidative mechanisms but can also interfere by mechanical hindrance with cellular and nuclear components, like, microtubules of the mitotic spindle [70, 71].

The majority of the NM tested both *in vitro* and *in vivo*, such as, metal nanoparticles [13, 72, 73] and both SW- and MWCNT [36, 74, 75] are able to cause DNA strand breaks or oxidative DNA lesions. Chromosome breakage and chromosome loss, the main genotoxicity endpoints, can be detected and quantified in target cells using the cytokinesis-block micronucleus method (CBMN assay), originated from acentric chromosome fragments or whole chromosomes lagging behind during cell division, coupled with the fluorescence *in situ* hybridization (FISH) analysis. DNA damage in peripheral blood leukocytes can be quantified by single cell gel electrophoresis (SCGE; Comet assay) a simple and sensitive method for detecting DNA strand breaks and alkali-labile sites, while oxidatively damaged DNA can be analyzed using the enzyme formamidopyrimidine glycosylase (FPG) and endonuclease III (endo III) which detect, respectively, oxidized purines and pyrimidines [75]. Although interactions between the particles and the assay cannot be totally excluded, the use of Comet assay in human-biomonitoring studies could provide valuable information for hazard identification of NMs [76]. Some studies showed dose-effect and dose-response relationships between the mass concentration of NM and the frequency of micronucleated lymphocytes, with a threshold for the above effects [72, 75].

DNA methylation, a major genomic mechanism of gene expression control, can be affected by reactive oxygen species (ROS), which are considered one of the main cellular stressors generated by PM exposure [77] as well as by some metals [78]. Conditions associated with reduced global DNA methylation content have been shown to interact with ambient PM

exposure to produce health-related outcomes. For instance, gene expression and the associated epigenetic changes have been demonstrated following exposure to metal-rich PM with aerodynamic diameters $<10\ \mu\text{m}$ (PM_{10}) in workers in an electric furnace [79].

MicroRNAs (miRNAs) are highly conserved, noncoding small RNAs that regulate the expression of broad gene networks at the posttranscriptional level [80] demonstrating that gold nanoparticles (AuNPs) altered the expression of 19 genes in human fetal lung fibroblasts, upregulating the microRNA-155 (miR-155), and downregulating the PROS1 gene. Silencing of miR-155 established PROS1 as its possible target gene. DNA methylation profiling analysis of the PROS1 gene revealed no changes in the methylation status of this gene in AuNP-treated fibroblasts, whereas chromatin condensation and reorganization were observed in the nucleus of fibroblasts exposed to AuNPs. These findings provide further insights into the molecular mechanisms underlying toxicity of AuNPs and their impact on epigenetic processes.

6. Biomarkers of Neurotoxicity

Existing research has shown that several NMs, like, quantum dots [81] and SWCNT [82] are capable of crossing the blood-brain barrier (BBB), damage it, and enter the brain. TiO_2 [83] and manganese oxide nanoparticles were capable of translocating along the olfactory nerve pathway to the brain after intranasal instillation exposure, accumulating in the olfactory bulb, cortex, and cerebellum [84]. The deposition in the brain of NMs such as, ferrous oxide [85] and CB [86] can stimulate oxidative stress, inflammatory responses, and pathological changes. The mechanisms of neuron injury are diverse for a wide variety of materials used and NMs produce different effects from environmental particles with potential effects both on peripheral and central nervous systems [87]. Adsorption and inhibition of acetylcholinesterase, a key enzyme present in the brain, blood, and nervous system, by SiO_2 , TiO_2 , Al_2O_3 , Al, Cu, carbon-coated copper, SWCNT and MWCNT suggest an interference of NMs with neurochemical processes and a use of AChE as a potential biomarker of neurotoxicity [88]. Dopaminergic neurons can be specifically targeted *in vitro* by a broad spectrum of NM, including Mn, Ag, or Cu nanoparticles [89]. *In vivo* studies showed SiO_2 -NPs entered into the brain and especially deposited in the striatum and induced oxidative damage and inflammation in dopaminergic brain areas with subsequent depletion of neuromediators [90].

Aerosolized welding fumes generated from two different welding processes, gas metal arc-mild steel (GMA-MS; low Mn, less water soluble), and manual metal arc-hard surfacing (MMA-HS; high Mn, more water-soluble) welding caused persistent molecular alterations in dopaminergic targets of treated animals [91]. To assess early effects from chemicals targeting the dopaminergic systems a neurochemical and neuroendocrine approach based on surrogate biomarkers in peripheral media has been previously developed [92]. Neurotoxicity of Mn to dopaminergic systems, investigated

in male workers occupationally exposed to Mn oxides containing fumes and metallurgic dusts, was revealed by increase in serum PRL levels [93].

It is unlikely that acute high dose causes CNS effects occur, whereas the long-term accumulation of low doses of ENP cannot be excluded; thus, the possibility that chronic exposures to NM can influence brain neurochemistry triggering or aggravating pathological processes cannot be ruled out [94].

7. Development of Biomarkers for Human Studies and Health Impact Assessment

The health effects of nanoparticles are, to a large extent, unknown, and currently there is no report of any definitive human disease that is caused by NMs exposure. Specific health end points on which future human studies should be focused are a controversial issue; as a result, studies on chronic effects with adequate latency are probably not feasible in the near future. In contrast, initial epidemiological studies (e.g., based on a cross-sectional design) could benefit of biomarkers to detect possible effects.

Therefore, there is a need for the selection of candidate biomarkers of early effects to be used in human studies.

The literature on short-term effect of air pollutants and the available literature on NMs suggests identifying multiple biomarkers, a biomarker profile, to assess both effects at the “portal of entry” (e.g., inflammatory changes, short-term respiratory changes, respiratory, eye or skin irritation) and systemic effects (e.g., heart-rate variability, platelet aggregation and prothrombotic changes, acute phase proteins) [95]. Biochemical tests or functional parameters to be assessed should be supported by consistent pathophysiological mechanisms, considering that the health end points ascribed to UFP, and possibly to NM, (cardiovascular, pulmonary, inflammatory) are often unspecific, show high prevalence in the general population, and share multiple nonoccupational risk factors [96].

Table 1 summarizes a panel of biomarkers of exposure and effect potentially available for human biomonitoring studies aimed at assessing early (i.e., subclinical) effects and health outcomes forerunners of disease endpoints. In bold are listed the more promising biomarkers (e.g., validated in human studies on people exposed to different ultrafine or fine particulates or fractions of them).

Since “nano-specific” biomarkers (or “nano-specific” effects) are difficult to demonstrate, practical considerations suggest to focus on the sensitivity instead of the specificity of biomarkers, to assess the causality of exposure conditions/scenarios and association with hazards (e.g., at workplace) but also to identify and validate, among a panel of already available biomarkers, the most suitable for BM studies in people exposed to NMs [97]. In spite of the advances made to ensure a higher degree of workers protection, epidemiological data specifically derived from nanoparticle-exposed humans, which should allow to validate biomarkers, are currently not available, owing to a number of issues, including the short latency since the introduction of NM

TABLE 1: Biomarkers of exposure and effect potentially available for human biomonitoring studies assessing exposure-effect relationships between particulate matter/ultrafine particles and health effects. In bold are listed the more promising biomarkers (e.g., validated in human studies on people exposed to different ultrafine or fine particulates or known fractions), whereas in *italic* are reported some potential biomarkers. Legend: EBC: Exhaled Breath Condensate; MDA: Malondialdehyde; T-BARS: Thiobarbituric Acid Reactive Substances; LTB₄: Leucotriene-B₄; NO: Nitric Oxide; 8-OH-dG: 8-hydroxy-2'-deoxyguanosine; 8-oxo-Gua: 8-oxo-7,8-dihydroguanine; 4-HNE: 4-hydroxy-2-nonenal; CC16: Clara Cell protein; hsCRP: high sensitivity C-reactive protein; IL-6: plasma Interleukin 6; sTNF-RII: soluble Tumor Necrosis Factor-receptor II; PAI-1: plasminogen activator inhibitor-1; V-CAM: vascular adhesion molecule; FPG-ENDOIII: lesions detected as sites in DNA sensitive to formamidopyrimidine DNA glycosylase and endonuclease III.

Exposure	Effective dose/early effect	Altered structure/function
(i) Exhaled particles in EBC (“deposited dose”)	(i) Lipid peroxidation products in EBC or blood (MDA, T-BARS, conjugated dienes, LTB ₄ , F2- and 8-isoprostane)	(i) <i>Fibrogenic markers</i>
(ii) <i>Circulating particles</i>	(ii) Oxidatively damaged DNA (8-OH-dG, 8-oxo-Gua)	(ii) <i>Cell transformation assays</i>
(iii) <i>Particle uptake by specialized cells (e.g., macrophages)</i>	(iii) Exhaled NO and nitrosative stress products (3-nitrotyrosine)	(iii) Micronucleus
(iv) Elemental analysis (for metallic NM, e.g., metal oxides) in biological fluids	(iv) Carbonyl compounds (4-HNE) in EBC	(iv) DNA strand breaks (Comet assay + FPG-ENDO III)
(v) Protein modification (“corona”)	(v) Serum pneumoproteins (CC16)	(v) DNA hypomethylation
	(vi) Platelet activation/aggregation and pro-thrombotic changes	(vi) MicroRNAs (miRNAs)
	(vii) Acute phase proteins (hsCRP), IL-6, and sTNF-RII	
	(viii) Clotting factors (fibrinogen, PAI-1), blood microparticles	
	(ix) <i>Tissue factor (TF) activity in plasma</i>	
	(x) Vascular adhesion molecules (V-CAM-1)	
	(xi) <i>Upregulation of GPIIb/IIIa in platelets</i>	

on the market, the overlap in exposure to combustion-derived UFP and engineered nanoparticles, the lack of standardized exposure metrics, the difficulty in identifying a target population with long, and consistent exposure leading to appreciable health effects [98].

In epidemiological research, BM is aimed at overcoming the growing frustration with the limitations of such crude markers of exposure as job titles, exposure registries or point estimates of airborne pollution. We should instead assess whether exposure to different NMs may lead to the same pathway for disease or share common mechanisms. But, to avoid “epidemiological frustrations” it is mandatory to identify at least intermediate biological changes of “acute” or “short-term” effects that could be indicative, if not predictive, of disease and apply them in cross-sectional investigations combining both exposure characterization to a given NM and sufficiently validated biomarkers.

For the NMs with known mechanisms or that share similar injury mechanisms (i.e., oxidative stress and inflammation, DNA damage) with UFPs, it is theoretically feasible to conduct biomarker studies starting with similar approaches; for example, to assess the early events associated with exposure to these NPs, biochemical changes indicative of local and systemic oxidative stress, systemic inflammation, and inflammatory response in target organs can be used [99]. Inflammatory biomarkers could be monitored in workers by using noninvasive methods (e.g., EBC collection). Pneumoproteins such as Clara cell protein (CC16) and surfactant-associated protein B (SP-B) in the serum have been validated as markers of alveolocapillary barrier integrity/permeability

in human studies on gaseous/particulate pollutants [100, 101]. Recently, CC16 determination in serum was studied in workers occupationally exposed to NM [102]. Besides CC16, the high-sensitivity Reactive C protein levels and inflammatory cell activation (increased ICAM-1 in macrophages) showed statistically significant changes among NM workers.

To date, exposure assessment and characterization represent the main issues in risk assessment; provided that exposure is, quantitatively or qualitatively, documented, then BM studies could be consistently implemented. Metal NPs which release metal ions or dissolve in biological media may be detectable (as metal species, not as particles) with appropriate analytical methods.

To give an estimate of deposited dose of particles by noninvasive techniques, Goldoni et al. [103] have developed a portable device designed to monitor the on-line exhalation kinetics of particles in a single breath and validated it in field studies of workers occupationally exposed to cristobalite in the size range of 0.3–2.5 μm [103] and to welding fumes [104]. Although the advantages of such an approach need further validation with different aerosols and model nanoparticles, the method suggests a tool to assess the dose of particles deposited within the respiratory tract.

By the breath analysis, tissue dose of pneumotoxic metals has been assessed in both workers occupationally exposed to hard metals (Co, Ti) [105], chromium(VI) [106]. Whether this technique is applicable to quantify the dose of metals eventually released by NMs deposited in the airways should be further assessed in field studies.

Although we are not able, at present, to identify “nano-specific” biomarkers that meet all the above criteria, we can use a battery of available biomarkers of local and systemic inflammation and oxidative stress to identify hazards and groups at risk. Certainly, it will take years to develop a panel of biomarkers suitable as indicators of exposure-specific disease outcomes, but relying on our knowledge from the air pollution studies, occupational exposures to particles and fibres of concerns, and advances in “-omics” techniques applied to nanotoxicology we can identify early biological responses related to injury pathways upon exposure to NMs.

Practical issues, including, cost, exposure levels (which are able to lead to “effective dose”), analytical requirements, and selection of an adequate sampling strategy, should be considered in every study. Biological materials should certainly be easy to obtain in sufficient amounts under routine conditions and without unacceptable discomfort and health risk for the individual or worker.

Evaluation of the possible health effects from exposure to NMs requires a stepwise approach considering the knowledge of the likelihood of a known exposure, tracking changes of such characterized exposure over time by exposure registries, conducting targeted epidemiological investigations, and possibly applying selected biomarkers [2, 24, 107, 108].

There are many efforts at international level to identify companies manufacturing NM and workers likely to be exposed to NMs, and some studies on occupational cohorts have been put in place [108–110]. The heterogeneity of NMs, making difficult to identify and recruit enough workers with the same exposure pattern, could be addressed by the use of biomarkers [98].

The feasibility of BM studies in NM workers should consider both conceptual and practical issues, such as: (i) data on the temporal trends in exposure levels; (ii) identification of parameters able to reflect internal exposure or early/reversible biological changes; (iii) specific analytical methods kept under control by quality assurance; (iv) establishing reference ranges and limit values which enable the interpretation of eventual changes; (v) assessment of the health significance and predictive value of observed changes.

Acknowledgment

This work supported by the EU-funded research project “Safe Nano Worker Exposure Scenarios”—SANOWORK (Grant no. N.FP7-280716).

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